In vitro analysis of the effects of water quality on Avian Influenza Viruses viability

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May 3, 2013

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Abstract

Avian-origin type A influenza viruses (AIV) are extremely variable. Much of the current research on type A influenza viruses has focused on transmission of the virus across bird species via direct contact. However, this experiment focuses on the water sources used by infected ducks as the source of infection. The objectives of this study were to examine the viability of low pathogenic avian influenza viruses (LPAIV) in the laboratory. As well as evaluating what environmental factors allow for LPAIV to have a higher viability in the environment. Furthermore, this research compared the viability of sporadically vs. frequently occurring LPAIVs isolated from the environment. The purpose of this study was to further analyze the effects of water quality on the viability of Avian Influenza Viruses. We demonstrated that more frequently isolated viruses showed a longer viability than less frequently occurring viruses under laboratory conditions. We also demonstrated that overall the viruses were most viable within the distilled water microcosms, which means that we may be overestimating the viability of AIV within water substrates.

Introduction

Avian-origin type A influenza viruses are extremely variable. The virulence of the virus and its transmissibility are due to two glycoproteins found on the capsule, hemagglutinin and neuraminidase (Perdue 2000). The hemagglutinin protein (HA) contains the receptor-binding site for the virus, which is key in establishing the virulence of the virus. The neuraminidase protein (NA) is involved with the release

of the virus particulates, the host cell, allowing for the spread of the influenza virus. There are 16 subtypes of HA and 9 NA, allow for a theoretical total of 144 hemagglutinin – neuraminidase combinations. Influenza A viruses can be separated into two distinct groups based on pathogenicity (Slemons et al. 1975). The virus is split into both high pathogenic and low pathogenic subtypes. The very virulent cases, restricted to H5 and H7 AIV, subtypes are termed as highly pathogenic avian influenza [HPAI], resulting in high mortality rates. All of the other subtypes are low pathogenicity avian influenza [LPAI] that cause mainly respiratory diseases that are much milder (Australian Wildlife Health Network 2011).

AIVs have 8 RNA segments, and only 2 RNA segments are used to determine the subtype of the virus; therefore, for example, 2 H4N6 LPAIVs may not be genetically identical, and thus could be very different and have different environmental tolerances. The ability of the virus to genetically reassort by recombination and mutation may contribute to the lethality of the virus (Webster et al. 1992).

Much of the current research on type A influenza viruses has focused on transmission of the virus within and across bird species via direct contact (Alexander 2000). Studies have determined that the virus could be sustained in wild bird populations by replicating in the intestines of the birds and thus also infect caged pet birds, poultry, and many avian species by direct contact.

Most evidence points to the idea that prevalence of influenza is caused by introduction of feral birds. This interaction is exemplified when captive-reared birds are moved to waterfowl migration routes. This understanding will hopefully allow

for better management strategies to be implemented and help prevent the spread of AIVs. Stallknecht et al. (1990a) found that the environment, specifically the water-organic matter interface, is a source of infection for ducks in their natural wetland habitats. However, the viruses cannot propagate in the environment and they can only degrade over time, because they do not have any host cells to infect. My experiment may serve as a bridge for research to begin to focus on water sources used by infected ducks, such as those that are important for migration, instead of looking at individual ducks as the primary sources of infection (Nolting and Slemons 2009). This is a very important finding because it will allow for further research to better understand the role of the environment in persistence and transmission of Avian Influenza Viruses.

Objectives

One objective of this study is to examine the persistence of LPAIV in different substrates including; distilled water, environmental water, and organic matter. A second objective of this study is to evaluate what environmental parameters allow for LPAIV to have a higher environmental persistence in the environment. A second objective of this project is to compare the environmental persistence of sporadically versus frequently occurring LPAIV 's. If the environmental tolerances of LPAIVs were better understood, important waterfowl habitat could be better managed and possibly reduce the level of environmental contamination/level of infective LPAIVs in the environment (Keeling, 2007). Furthermore, if the tolerances of ubiquitous and sporadic virus strains found within the environment were compared in the water conditions and the organic matter conditions, then we hoped to find that the

frequently occurring virus strains persist in the organic matter longer due to higher environmental tolerance than the sporadic strains found in the environment.

I hypothesized that if more frequently occurring LPAIV 's persist longer in the environmental microcosms, organic matter in particular, when compared to the less frequently isolated virus strains from year to year. The third objective of this project was to further examine how closely environmental conditions could be simulated in the laboratory microcosms.

This work is relevant and important to gaining understanding of the role of indirect transmission of LPAIV 's through the environment and its potential adding to spread avian influenza. Little research has been conducted on infection due to indirect exposure to the virus via environmental media. Knowledge of the environmental tolerances of LPAIV is very limited, which was the focus of this research. The possibility of better managing wetland conditions after this experiment could be to promote conditions that are not favorable to persistence of the virus (Lewis 1995).

Little research has been conducted on infection due to indirect exposure to the virus by environmental means (Hinshaw 1979). Research concerning LPAIV persistence in the environment is an ever-growing field and there is much room for progress. Low pathogenic Avian Influenza Virus's, LPAIV 's, are extremely variable and are known to have caused all of the major avian influenza pandemics in the history of humans (Olsen 2006). The first documented outbreak of "fowl plague" occurred in 1878 but the common etiology of avian and mammalian influenza, the type A influenza virus, was not demonstrated until 1955. The worst influenza

pandemic in the history occurred in 1918, when a H1N1 subtype avian-origin type A influenza virus jumped the species barrier into people. Known as “Spanish Flu”, this novel virus caused the death of an estimated 30-50 million people worldwide.

The persistence of Avian Influenza in water has also been recently studied (Stallknecht, et al. 1990a). For example, the differences in virus persisting differ significantly at the different water temperatures ranging from 17 °C and 28 °C, modeling both winter and summer temperatures respectively. The virus persisted, at 17 °C modeled environmental winter temperatures, longer for a period of 126 to 207 days compared to the persistence of infectivity from 30 to 102 days during the modeled summer temperature of 28 °C. Indicating the ability of viruses to overwinter in frozen ponds or waterways, allowing the virus to be return to local breeding areas in the spring and continue being infectious. The decreased persistence of AIVs at higher water temperatures may also indirectly suggest enhanced virus survival during the winter. The high temperatures may limit transmission among species during the summer allowing for an increase in susceptible birds for transmission during the winter. However, these results are limited because they were only carried out under laboratory conditions and cannot be extended to AIV persistence in water under natural conditions.

Another study conducted by Stallknecht et al. (1990) examined the effects of pH, temperature, and salinity on the persistence of the avian influenza viruses in water. They consistently found that the duration of infectivity of AIV decreased with increased salinity and pH. The persistence of the virus was the longest under the experimental conditions at pH 6.2 with salt (20ppt) and pH 8.2 without salt. These

findings have application to field conditions, with salinity at 0 ppt being freshwater and at 30 ppm being the saltwater, these results could be applied to managing the environmental parameters of an aquatic habitat of wild avian populations as the potential areas for viral transmission.

The environmental parameters that have previously been studied in the lab include temperature, pH, and salinity. Other environmental factors that have been suggested for further research are conductivity, oxidation-reduction potential, and dissolved oxygen content. A recent study conducted by Shahid (2009), tested at three different temperature, pH levels, UV lighting, and a variety of commercially available detergents on the persistence of the H5N1 AIV, is a highly pathogenic virus. The temperature and pH level findings were consistent with other studies, inactivated faster at higher temperatures and higher pH, the UV lighting was ineffective and never inactivated the virus completely in an hours time, and the commercial soaps inactivated the virus at recommended concentrations in parts per million.

My experiment is a continuation of a previous honors student's (Schwarten, unpublished) study, which tested for the environmental persistence of LPAIVs in different substrates and worked on developing a model within the lab for environmental substrates (Schwarten 2009). The previous study found that the virus subtype H4N6 could persist for at least 28 days in an environmental model simulating summer conditions. The data suggests environmental substrates and temperature do not have any interactive effects on virus persistence, which allowed for my study to examine each treatment individually. My studies conducted in Dr.

Slemon's Lab examined virus persistence that occur sporadic versus frequently. Additionally, my studies were conducted for 72 days versus 28, which allowed us to reach an endpoint for virus persistence. We also analyzed the effects of water quality on virus viability, critiqued our sampling methods between trials one and two of my research, and assessed how well the laboratory microcosm conditions simulated the actual environmental conditions at Winous Point Marsh Conservancy.

Methods

To further explore how environmental factors affect the persistence of low pathogenic avian – origin type A influenza viruses (LPAIV 's), this project began with a series of two separate eight-week experiments. The water samples were taken from Winous Point Marsh Conservancy, located along the Lake Erie shoreline in northwest Ohio (Figure 2). The environmental water and organic matter samples for trial one were taken from Horseshoe Island on July 4, 2011. The environmental water and organic matter samples for trial two were taken from Norton's Ditch on June 7, 2012. The length of the time period for the project was selected to show varying persistence in the virus strains between the environmental microcosms of water and organic matter and the distilled water microcosms.

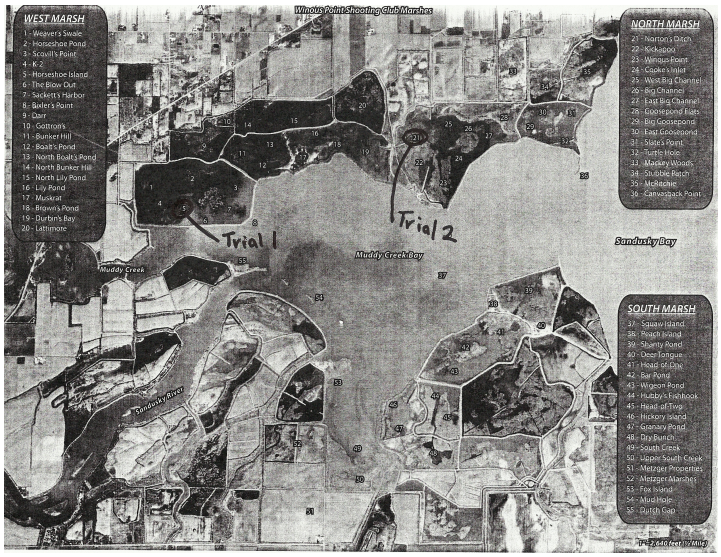

Figure 2. Winous Point Marsh Conservancy environmental sampling sites for trials one and two of research.

Laboratory Model System

We constructed a series of microcosms to study three different conditions: distilled water, environmental water, and organic matter. All of the samples were inoculated with 1 x 10 ^6.5 EID50/ 1 mL amount of virus infectivity, allowing for accurate calculations to determine the infectivity of the virus over the course of the experiment by using the 50% egg infective dose (EID50). In the first round of experiments, six viruses were selected, three being frequently isolated strains and the other three were less frequently isolated strains from the Winous Point Marsh Conservancy. The viruses were chosen by Dr. Slemons based on their frequency of isolation determined by his surveillance program, which has been running since 1986, are H2N2, H8N4, H5N1; less frequently isolated strains, and H4N6, H6N2, H11N9; being the more frequently isolated strains (Slemons 1974). Virus 1, H2N2, was isolated in November of 2007 from *Lophodytes cuculattus* at Winous Point Marsh Conservancy. Virus 2, H8N4, was isolated in October of 2007 from *Anas*

discors at Magee Marsh. Virus 3, H5N1, was isolated in August of 2004 from *Aix sponsa* at the Darvy Unit of Ottawa National Wildlife Refuge. Virus 4, H6N2, was isolated in October of 2010 from an *Anas americana* at Winous Point. Virus 5, H11N9, was isolated from North Lilly at Winous Point during August from the summer of 2010 from Anas platyrhynchos. Virus 6, H4N6, was isolated in August of 2010 as well from North Lilly at Winous Point from Anas platyrhynchos.

In the first trial two microcosms were constructed for each virus; one with the simulated environmental conditions and one microcosm inoculated filled with only distilled water. In the second round of experiments I evaluated the first round methods to obtain more detailed data using the same viruses. I separated the environmental water and organic matter into two separate microcosms for more accurate sampling measurements demonstrating the difference between the water and organic matter substrates. A total of five type A avian influenza viruses were selected by Dr. Slemons for the second trial, which were the same virus isolates from the first trial just without H4N6. The frequently isolated viruses selected were H6N2 and H11N9. The less frequently isolated viruses selected were H2N2, H8N4, and H5N1 from previously collected environmental samplings.

Virus	Virus 1	Virus 2	Virus 3	Virus 4	Virus 5	Virus 6
Subtype	H2N2	H8N4	H5N1	H4N6	H6N2	H11N9
Location	Winous Point	Magee Marsh	Ottawa Wildlife Refuge	Winous Point	Winous Point	Winous Point
Species	Lophodytes cuculattus	Anas discors	Aix sponsa	Anas americana	Anas platyrhynchos	Anas platyrhynchos

Table 1. Virus isolate descriptions.

After these viruses were selected the stock viruses were prepared similarly for both trials. First the first passage avian influenza virus (AIV) isolate

chorioallantoic fluid (CAF) was obtained from freezer storage and thawed until the fluid became slightly slushy. Approximately 0.5 mL of CAF was drawn up through a 26-gauge needle into a 3 mL syringe to break up the virus particle clumps. The CAF was then passed through a syringe, filtered, and added to 1.8 mL Brain Heart Infusion Broth (BHIB) containing penicillin and streptomycin and mixed thoroughly making a 10^{-1} dilution stock. Serial dilutions to 10^{-4} were prepared for each isolate. Six 10-day-old embryonating chicken eggs were inoculated with 0.1 mL of each 10^{-2} (1:100) and 10^{-4} (1:10,000) dilution stock and incubated for 48 hours at 95 °F and then chilled overnight at 4 °C. All available CAF was then harvested from each egg separately and tested for hemaggluination (HA) and an HA titer was determined. The CAF was obtained and thawed to determine the EID_{50} of the stock viruses and then filtered as if preparing the stock virus and 0.2 mL of CAF was added to 1.8 mL of BHIB for serial dilutions 10^{-1} to 10^{-9}. 0.1 mL of dilution stock was inoculated into four 10-day-old embryonating chicken eggs, incubated for 48 hours, and then chilled overnight at 4 °C. Each egg was tested for HA activity and the presence of type A influenza virus was tested for using Flu Detect. The EID_{50} for each viral isolate was calculated using the Reed and Muench method. All of the fluid with an HA titer ≥ 32 from 4-6 eggs was pooled and aliquoted into at least four 3.6 mL cryogenic vials and four 1.8 mL cryogenic vials, labeled, and frozen at -80 °C until further use.

The microcosms were then constructed for each trial. The containers were washed with soap and warm water and rinsed with distilled water. The negative control microcosm was filled with 1500 mL and 500 mL for trials one and two, respectively, and the water level was marked with a permanent marker to monitor

the water level and evaporation. The microcosm lid was replaced, snapped closed, and labeled with a sharpie marker. The distilled water microcosms were filled with 1500 mL and 500 mL for trials one and two, respectively, the water level was marked with a sharpie, the lid was snapped closed, and the microcosm was labeled. The environmental water and organic matter substrate microcosms were constructed by placing 500 mL moist environmental organic matter into the microcosms. In the first trial the organic matter and 1500 mL of environmental water were placed in the same microcosm container. In the second trial two separate microcosms were constructed for 500 mL of organic matter and then 500 mL of environmental water were made. The substrate level was marked with a sharpie, the microcosm was snapped shut, and labeled. The microcosms were then placed on the shelves of a tissue culture incubator that had been adjusted to 28 °C.

We attempted to keep all the microcosms at a constant temperature for the duration of the experiment. The temperature used was determined by our field data gathered during the summer at the Winous Point Marsh Conservancy, we decided to use 28 °C as the baseline temperature for all of the microcosms to recreate the summer conditions when environmental samples were collected during. After the microcosms had been constructed they were then inoculated with the select viruses. The virus inocula were first thawed and then placed one at a time in the biosafety cabinet with a syringe, needle, and tongue depressor. The microcosm was retrieved from the tissue culture incubator and placed in the biosafety cabinet. The microcosm was opened and inoculated with a $1x10^8$ AIV/mL chorioallantoic fluid equivalent into 1500 mL microcosms for the first trial and $1x10^5$ AIV/mL

chorioallantoic fluid equivalent into 500 mL microcosms for the second trial. The microcosms were then stirred with a tongue depressor to homogenize the microcosms. The tongue depressor was then disposed of and, the inoculated microcosm was closed and the container was sprayed, and then removed from the biosafety cabinet back to the tissue culture incubator.

Environmental Tolerance

The sampling scheme used during this experiment included sampling once a week for 8 weeks; a total of nine sampling periods from t0 to t8 weeks, were completed on two different days of the week in order to minimize contamination when sampling the environmental and distilled water microcosms. The inoculated microcosm was retrieved from the tissue culture incubator, placed within the biosafety cabinet, and disinfected with ethyl alcohol prior to opening. For both rounds of experiments the water abiotic parameters that we tested for twice weekly included temperature, pH, salinity, conductivity, dissolved oxygen, and oxidation-reduction potential, which were measured with a YSI 556 multi-parameter meter that was calibrated weekly. The organic matter abiotic parameters, including temperature, pH, and oxidation-reduction potential, were measured with a Hanna HI 991003 pH/ORP/temperature meter. The inoculated microcosm was opened and the dissolved oxygen probe was inserted into the water only. The dissolved oxygen probe has a delicate reading lense and was not used in the organic matter substrates. I proceeded with measuring the temperature, pH, and ORP of water with pH probe. The readings along with noting any changes in color/opacity of water was recorded on the microcosm sheet.

After measuring the water and soil parameters as mentioned previously disposable transfer pipets and tongue depressors were used to obtain water and organic matter samples, respectively, from the microcosms and placed in 1.8 ml sterile cryovials. The cryovial with 0.9 mL of BHIB was opened and using a disposable transfer pipette or tongue depressor substrate was transferred from the microcosm to the cryovial. The transfer pipette was disposed of and the cryovial was closed. The microcosm container and biosafety cabinet were disinfected between microcosms to eliminate cross-contamination. One vial for each virus in every microcosm and substrate (6 distilled water samples, 6 environmental water samples, 6 organic matter samples, and 1 control sample for the first round of experiments versus 5 distilled water samples, 5 environmental water samples, 5 organic matter samples, and 1 control sample for the second round of experiments). The samples taken were about 0.9 mL added to a prefilled vial of 0.9 mL of BHIB, which were all then stored in a -86 °C freezer to until EID 50 assay was calculated. The samples collected were tested for the presence of LPAIV 's first to help determine if the viruses have a preference for any of the following microenvironments: distilled water, organic matter, or environmental water.

This project also utilized low pathogenic subtypes to determine the infectivity of the virus over time by using the 50% egg infective dose (EID). First, specific free pathogen eggs were incubated for 10 days. Then the eggs were inoculated with the samples taken during the experiment and then 3 days later an EID 50 was run to test for the presence of virus.

The data generated from the microcosms was analyzed statistically using the program R. The data was also evaluated for normality, examined graphically, and compared qualitatively following the analysis to further evaluate the sources of variation between substrates, viruses, water parameters, and the trials.

Results

The results of egg inoculation following sampling did not show virus viability consistency between trials 1 and 2 (Table 2). The viruses are grouped by sporadic occurrence in the environment, viruses 1-3, and more frequent occurrence in the environment, viruses 4-6. Virus 1, H2N2, persisted longer in the environmental substrate than distilled water in both trials. Virus 2, H8N4, persisted the longest in the distilled water microcosm. Virus 3, H5N1, persisted the longest in the distilled water as well. After reviewing the data for the ubiquitous viruses, I found that virus 4, H6N2, persisted the longest in the distilled water microcosm. Virus 5, H11N9, persisted the longest in the distilled water microcosm in trial one but the longest in the environmental water microcosm in trial two. Virus 6, H4N6, lasted equally long in the distilled water and environmental water microcosms in trail one. On average, viruses lasted for 4.7 weeks in trial 1 and 3.5 weeks in trial 2 in distilled water microcosms. In environmental water microcosms viruses lasted for 2.7 weeks in trial 1 and 4.2 weeks in trial 2. In organic matter microcosms, viruses were viable for 2 weeks in trial 1 and 2.4 weeks in trial 2. Sporadic viruses persisted an average of 3.67 weeks in both trials one and two and the ubiquitous viruses persisted an average of 4 weeks in trial one and 4.5 weeks in trial two. We were unable to

compare virus history in this project due to viruses being from different species altogether or not being included in both trials.

Trial		Egg Inoculation			PCR								
	Virus	DH	EH	OM	Week 0			Week 3			Week 7		
Trial 1					DH	EH	OM	DH	EH	OM	DH	EH	OM
	Virus 1	1	1	2	+	-	-	-	-	-	-	-	-
	Virus 2	6	1	3	+	+	+	+	-	-	-	+	-
	Virus 3	6	4	1	+	+	-	+	-	+	-	+	-
	Virus 4	6	4	1	+	+	-	-	-	-	-	+	-
	Virus 5	5	1	2	+	+	-	-	-	-	-	-	-
	Virus 6	4	4	3	+	+	+	-	-	-	-	-	-

Table 2. Egg inoculation results showing virus viability through weeks and PCR results.

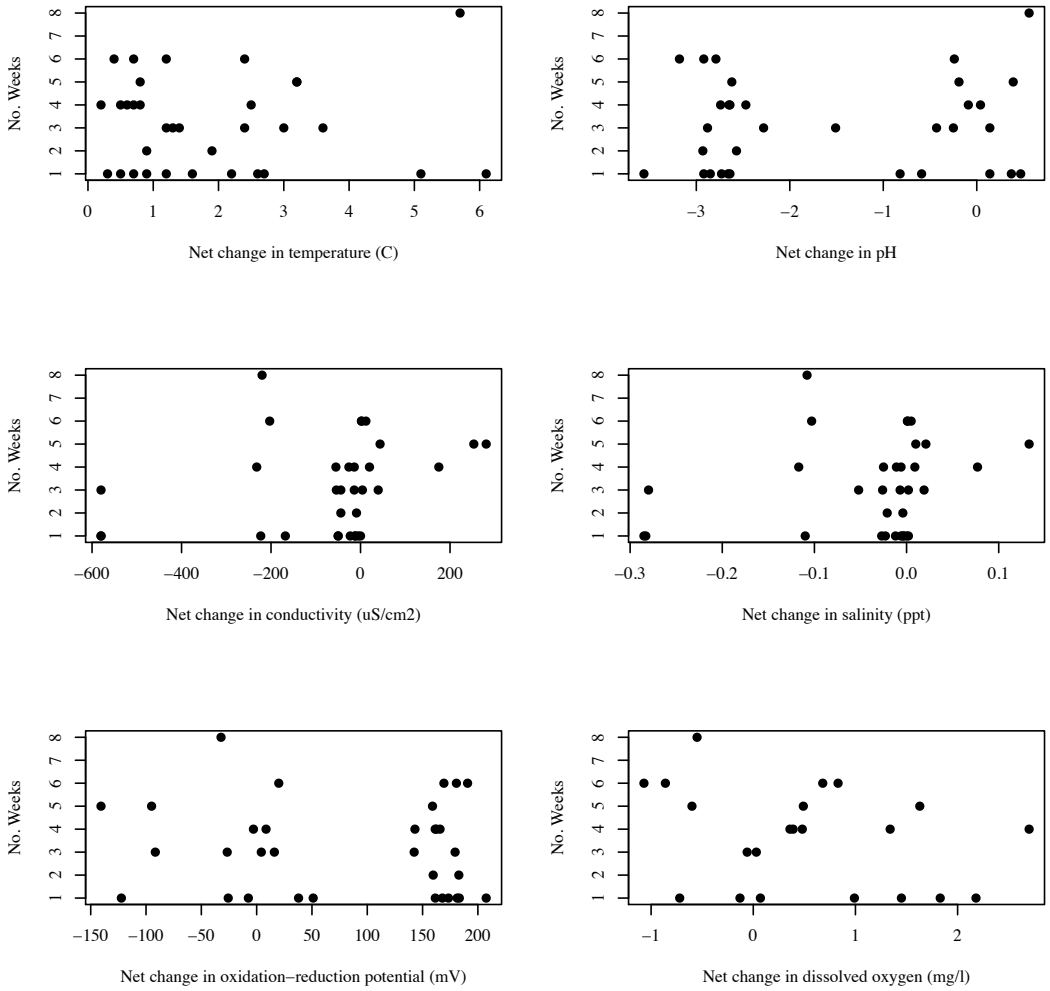

Figure 3. Virus persistence related to water chemistry parameters.

Parameter	Winous Point Median	Laboratory Median
Water temperature (°C)	28.06	28.5
Water pH	8.5	6.6
Water conductivity (μS/cm^2)	2914	175
Water salinity (ppt)	1.33	0.04
Water redox potential (mV)	-132.6	68.3
Water dissolved oxygen (mg/L)	0.71	5.56
Organic Matter temperature (°C)	27.2	28.1
Organic Matter pH	7.4	6.89
Organic Matter redox potential (mV)	-70	36.2

Table 3. Winous Point and laboratory water parameters.

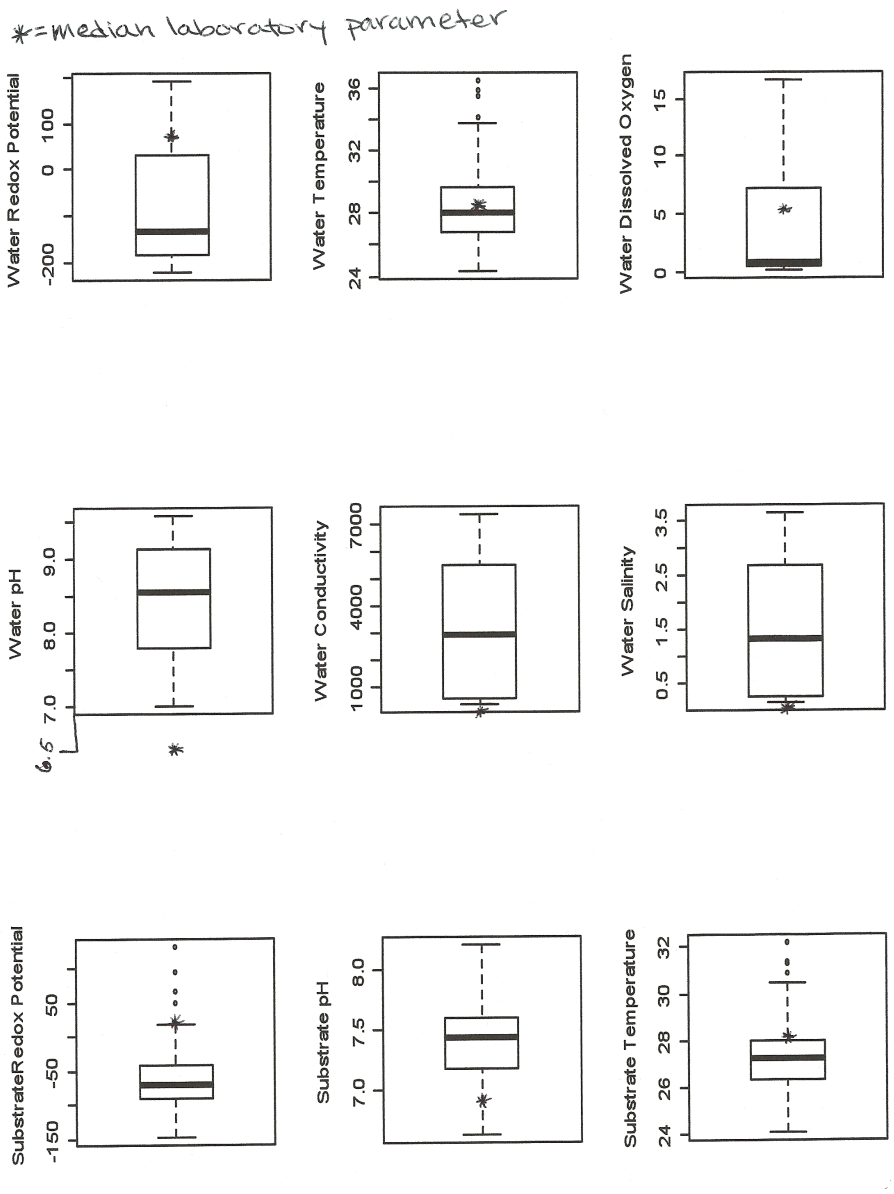

Figure 4. Winous Point and laboratory water parameter comparison.

Discussion

After a review of the data collected over this study, I found that viability between the trials and within treatments was different. There was no repeatability between trials however, which leads us to not reject our null hypothesis. Furthermore, there is a difference in environmental persistence between the

ubiquitous and sporadic virus strains that we tested under simulated environmental conditions within the laboratory. Our distilled water substrate will allowed us to validate our study using previous studies in the literature. This experiment also hypothesized that we would see longer virus persistence in the organic matter microcosms. However, this was not the case and in seven out of the eleven viruses, the virus persisted for the greatest duration of time in the distilled water microcosms. This experiment may also began to address the question of as to whether distilled water microcosms are relevant to a natural system, but only when used with LPAIVs and conditions similar to Winous Point Marsh Conservancy. Considering egg inoculation results between trials, we discovered that we had "skip weeks", when there was no virus found in the previous week but then found again later through out testing. These results could be due to the sampling technique. With the larger sized microcosms, 1500 mL and 500 mL respectively for trial one and two, compared to the previous experiment conducted Schwarten (unpublished), 35 mL microcosms, it is possible that there could have been virus present in the larger microcosms however when only 0.9 mL samples were taken the virus could have been missed. We further confirmed the presence or absence of viruses with PCR (polymerase chain reaction) testing after specific weeks of trial one. We tested PCR after week seven and found three positive tests for environmental water viruses 2, 3, and 6. However, virus had not been found positive in these samples since week 1, 4, and 4 respectively. The PCR had also not been found positive for these samples since time zero right after the initial inoculation of the experiment. Again, this finding does not support the sporadic v. ubiquitous virus hypothesis. Two of the

three viruses, which showed nucleic acid or RNA after PCR testing after week 7, were sporadically isolated strains (Table 2). However, just because the samples tested positive for PCR this does not mean that the virus was infective here.

Further comparison of Schwarten's data and my own is what really fueled the intense procedure and sampling method critiquing. Schwarten had issues with his microcosms becoming anoxic too quickly and evaporation. So during my experiments we used microcosms that had greater air surface space in larger square containers instead of conical vials. This allowed us to neglect the need to worry about evaporation altogether. Also we lengthened the time of my experiment to find endpoints for virus persistence since Schwarten was unable to find them during his four-week experiment. Schwarten also started out with inoculating a higher infective dose into his microcosms at the start of his experiment than I was able to given the limited amounts of original stock virus.

A closer look into the data collected concerning the water quality parameters and the virus persistence may be attributed to changes in water pH, temperature, oxidation-reduction potential, or conductivity. Any water quality parameters that are connected to the persistence of LPAIVs could, as I mentioned previously, be managed to promote the conditions that are not favorable to the persistence of the virus after further research.

Between trial one and trial two, a few changes were made to the experiment to account for previous pitfalls. Smaller amounts of substrate were used to decrease the number of skip weeks during trial two. Instead of 1500 mL of substrate, 500 mL were used and the substrate was stirred with a wooden tongue depressor prior to

sampling to break up virus clumps. This improved the success of collecting virus in trial two. Trial 1 had 10 total skip weeks between the six viruses that occurred in five out of the six viruses. Trial 2 had 7 skips that occurred in 4 out of the 5 viruses. Additionally, in trial two the environmental water and organic matter were placed into two separate microcosms to further differentiate between the environmental substrates. The water parameters and water chemistry were held constant between trials one and two at the start of incubation. For the second trial, the water-parameter meter was calibrated each week to ensure the data was not skewed by a technological error. Overall, the improvements made in the inoculation and sampling procedure protocol were demonstrated in the reduced number of skips, increased sampling consistency, and data collection in trial two.

This study presents a few different pitfalls that must be addressed. The first is that the study is only covering a snapshot in time. That data collected will be from one or a few specific time period(s). This allows for only an incomplete picture concerning the overall survivability of avian influenza virus in the environment.

Knowing that temperature is a significant factor in determining how long the virus will remain viable in the environment, the data collected from this study will only be relevant to current conditions (Stallknecht et al. 1990). Separate studies will need to be conducted during the different seasons to determine any correlations or differences between the seasons. Based on the current research, environmental persistence would likely increase in the colder months, and then decrease in the warmer months. We chose to work with summer conditions in this study because they are supposedly the most inhospitable conditions for the virus.

My study, while using 6 different LPAIVs, is only using 6 LPAIVs. Therefore, there is a tremendous amount of variation in the influenza virus landscape that we are not including in this experiment like other subtypes or genotypes. Also, we have no way of measuring the biotic aspect of this study. We know that there's plant life, bacteria, and protozoa living in our environmental substrates, but we have no way of characterizing them or measuring them.

The construction of the microcosm, inoculation procedure, and the sampling protocol need to be further critiqued in order to evaluate whether or not the environmental parameters are being effectively simulated under laboratory conditions. Table 2 and table 3 show that while the temperature and pH levels were able to be accurately simulated close to environmental conditions in the lab, there are still some conditions that we have yet to account for within a changing environment. For instance, the presence of microorganisms and nonorganic particulate matter levels may also play a role in the hospitality to viability. Further investigation into the virus selection process and procedure protocol could increase the repeatability and success of the trials. Using smaller microcosm containers with a higher virus infectivity to be sampled every day instead of on a weekly basis could further this research. The smaller containers would help us isolate the virus during sampling and hopefully increase our chances of finding when it actually drops out of the environment. Future experiments could also focus on further differentiating between frequently isolated and sporadically isolated viruses to understand how water chemistry favors the maintenance of viruses in the environment. Additionally, Keeler et. al (2013) found that the effects of subtype and genotype of the virus also

effect AIV persistence in aquatic habitats. Viruses more similar in subtype and genotype should be selected to limit uncontrolled factors. Focusing on refining the virus sampling and protocol procedure will increase the success, reproducibility, and reliability of future research and experiments.

Acknowledgments

This research project was undertaken by myself in partial fulfillment for my Honors Degree Bachelor of Science from The Ohio State University with the help of Dr. Robert Gates, from The Ohio State School of Environment and Natural Resources, Charles Schwarten, from The Ohio State College of Veterinary Medicine, Jacqueline Nolting, from The Ohio State College of Veterinarian Preventative Medicine, and Dr. Richard Slemons, from The Ohio State College of Veterinary Medicine.

Literature Cited

Alexander, Dennis J. "A Review of Avian Influenza in Different Bird Species." *Veterinary Microbiology* 74 (2000): 3-13.

Austrailian Wildlife Health Network. Avian Newcastle Disease and Australian Wild Birds Fact Sheet. (2011). http://www.wildlifehealth.org.au/AWHN_Admin/ManageWebsite%5CFactSheets%5CUploadedFiles/118/Newcastle%20Disease%2019%20Jul%202011%20(1.1).pdf.

Hinshaw, V. S., R. G. Webster, & B. Turner. 1979. Water-bourne transmission of influenza A viruses. Intervirology 11: 66-69.

Keeler, S.P., Lebarbenchon, C., & Stallknecht, D.E. 2013. Strain-related variation in the persistence of influenza A virus in three types of water: distilled water, filtered surface water, and intact surface water. *Virology Journal* 10(38). http://www.virologyj.com/content/10/1/13.

Keeling M. J. & P. Rohani. 2007. Modeling infectious diseases. Princeton University Press, Princeton, NJ.

Lewis, W.M. 1995. Wetlands: characteristics and boundaries. National Academies Press, Washington, DC. pp. 121.

Nolting, J. M. & R.D. Slemons. 2009. Phenotypic variations in low pathogenic H1N1 avian influenza viruses resulting from genotypic differences among isolates. The 7th International Symposium on Avian Influenza Poster Presentation. April, 2009, Athens, GA.

Olsen, B., V. J. Munster, A. Wallensten, J. Waldenstrom, A. D. M. E. Osterhaus, and R. A. M. Rouchier. 2006. Global patterns of influenza A virus in wild birds. Science 312: 384 – 388.

Perdue, Michael L., David L. Suarez. "Structural Features of the Avian Influenza Virus Hemagglutinin that Influenza Virulence". *Veterinary Microbiology* 74 (2000): 77-86.

Schwarten, C., Slemons, R., Gates, R. & Nolting J. (2009). "Environmental tolerance of a H4N6 avian-origin type A influenza virus". Unpublished.

Shahid, Muhammad A., Abubakar, Muhammad, Hameed, Sajid, & Hassan Shamsul. 2009. "Avian Influenza Virus (H5N1); Effects of Physico-chemical Factors on its Survival." *Virology Journal* 6(38). http://www.virologyj.com/content/6/1/38.

Slemons, R.D. & Easterday, B.C. 1975. The natural history of type A influenza viruses in wild waterfowl. *In* Third international wildlife disease conference, L.A. Page (ed.). Plenum Press, New York, New York. pp. 215-224.

Slemons, R.D., D.C. Johnson, J.S. Osborn, & F. Hayes. 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Diseases 18: 119-124.

Stallknecht, D. E., M. T. Kearney, S. M. Shane, and P. J. Zwank. 1990. Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. Avian Diseases 34: 412-418.

Stallknecht, D. E., S. M. Shane, M. T. Kearney, and P. J. Zwank. 1990a. Persistence of avian influenza viruses in water. Avian Diseases 34: 406-411.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. Microbiological Reviews 56: 152-179.